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Molecular detection of pathogens in water – The pros and cons of molecular techniques

Rosina Girones [a,*], Maria Antonia Ferrús [b], José Luis Alonso [c], Jesus Rodriguez-Manzano [a], Byron Calgua [a], Adriana de Abreu Corrêa [a,d], Ayalkibet Hundesa [a], Anna Carratala [a], Sílvia Bofill-Mas [a]

[a] Department of Microbiology, Faculty of Biology, University of Barcelona. Av. Diagonal 645, 08028 Barcelona, Spain
[b] Department of Biotechnology, Polytechnic University of Valencia, Camino de Vera 14, 46022 Valencia, Spain
[c] Institute of Water Engineering and Environment, Polytechnic University of Valencia, Camino de Vera 14, 46022 Valencia, Spain
[d] Department of Microbiology and Parasitology, Laboratory of Applied Virology, Federal University of Santa Catarina, 88040-900 Florianópolis, Santa Catarina, Brazil

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ABSTRACT

Pollution of water by sewage and run-off from farms produces a serious public health problem in many countries. Viruses, along with bacteria and protozoa in the intestine or in urine are shed and transported through the sewer system. Even in highly industrialized countries, pathogens, including viruses, are prevalent throughout the environment. Molecular methods are used to monitor viral, bacterial, and protozoan pathogens, and to track pathogen- and source-specific markers in the environment. Molecular techniques, specifically polymerase chain reaction-based methods, provide sensitive, rapid, and quantitative analytical tools with which to study such pathogens, including new or emerging strains. These techniques are used to evaluate the microbiological quality of food and water, and to assess the efficiency of virus removal in drinking and wastewater treatment plants. The range of methods available for the application of molecular techniques has increased, and the costs involved have fallen. These developments have allowed the potential standardization and automation of certain techniques. In some cases they facilitate the identification, genotyping, enumeration, viability assessment, and source-tracking of human and animal contamination. Additionally, recent improvements in detection technologies have allowed the simultaneous detection of multiple targets in a single assay. However, the molecular techniques available today and those under development require further refinement in order to be standardized and applicable to a diversity of matrices. Water disinfection treatments may have an effect on the viability of pathogens and the numbers obtained by molecular techniques may overestimate the quantification of infectious microorganisms. The pros and cons of molecular techniques for the detection and quantification of pathogens in water are discussed.

Abbreviations: MST, microbial source-tracking; HAdV, human adenoviruses; HAV, hepatitis A virus; HEV, hepatitis E virus; JCPyV, human polyomavirus JC; BKPyV, human polyomavirus BK; PCR, polymerase chain reaction; qPCR, quantitative PCR; qRT-PCR, quantitative reverse transcriptase PCR; NASBA, acid sequence-based amplification; CFU, colony-forming units; mPCR, multiplex PCR; IFAs, immunofluorescent assays; IMS, immunomagnetic separation; RT-PCR, reverse transcriptase PCR; mRNA, messenger RNA; PAdV, porcine adenoviruses; BPyV, bovine polyomaviruses; EMA, ethidium monoazide; PMA, propidium monoazide; VBNC, viable non-culturable; PBS, phosphate buffered saline; nPCR, nested-PCR.

* *Corresponding author*. Tel.: +34 93 402 1483; fax: +34 93 403 9047.
E-mail address: rgirones@ub.edu (R. Girones).

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Contents

1. Introduction

Significant numbers of human microbial pathogens are present in urban sewage and may be considered environmental contaminants. Although most pathogens can be removed by sewage treatment, many are discharged into the effluent and enter receiving waters. Point-source pollution enters the environment at distinct locations, through a direct route of discharge of treated or untreated sewage. Non-point sources of contamination are of significant concern with respect to the dissemination of pathogens and their indicators in the water systems. They are generally diffuse and intermittent and may be attributable to the run-off from urban and agricultural areas, leakage from sewers and septic systems, and sewer overflows (Stewart et al., 2008). Molecular methods are used to monitor viral, bacterial and protozoan pathogens, and to track pathogen- and source-specific markers in the environment.

Classic microbiological indicators such as faecal coliforms, *E. coli* and *Enterococci* are the indicators most commonly analyzed to evaluate the level of faecal contamination. They are also used to assess the efficiency of pathogen removal in water purification processes. However, whether these bacteria are suitable indicators of the occurrence and concentration of human viruses and protozoa cysts has been questioned (Lipp et al., 2001; Tree et al., 2003; Wéry et al., 2008). Indicator bacteria are more sensitive to inactivation through treatment processes and by sunlight than viral or protozoan pathogens (Hurst et al., 2002; Sinclair et al., 2009). Other limitations have been associated with their application: short survival compared to pathogens (McFeters et al., 1974), non-exclusive faecal source (Scott et al., 2002; Simpson et al., 2002), ability to multiply in some environments (Solo-Gabriele et al., 2000; Pote et al., 2009), inability to identify the source of faecal contamination (point and non-point) (Field et al., 2003) and low correlation with the presence of pathogens (Pina et al., 1998; Horman et al., 2004; Savichtcheva and Okabe, 2006). As a result, none of the bacterial indicators currently used meets all established criteria for water quality. Thus in certain cases, such as drinking or bathing water, direct analysis of specific pathogens of concern is considered to be a more suitable alternative.

Source water contamination by *Cryptosporidium* and *Giardia* presents a particular challenge to water-quality managers for several reasons. These include the ubiquity of protozoa in wastewater effluents (Carey et al., 2004), the widespread infection of domestic animals and wildlife (Fayer, 2004), the resistance of protozoans, especially *Cryptosporidium*, to traditional disinfection methods (Steiner et al., 1997), and the uncertain relationship between the presence of protozoans and faecal indicator bacteria typically used in water quality monitoring (Chauret et al., 1995; Cizek et al., 2008; Keeley and Faulkner, 2008).

Molecular techniques, specifically nucleic acid amplification procedures, provide sensitive, rapid and quantitative analytical tools for detecting specific pathogens, including new emergent strains and indicators. They are used to evaluate the microbiological quality of food and water, the efficiency of virus removal in drinking and wastewater treatment plants, and as microbial source-tracking (MST) tools (Albinana-Gimenez et al., 2009b; Field et al., 2003; Hundesa et al., 2006).

2. Pathogens in water

2.1. *Viruses*

The list of potentially pathogenic viruses present in urban sewage includes the DNA viruses, adenovirus and polyomavirus, and RNA viruses such as enterovirus, hepatitis A and E viruses, norovirus, rotavirus and astrovirus. Human

adenoviruses (HAdV) and polyomaviruses exhibit a high prevalence in all geographical areas studied (Pina et al., 1998; Bofill-Mas et al., 2000). Enteroviruses, noroviruses, rotavirus, and astroviruses have been described as showing diverse prevalence levels, depending on the time of year and the presence of outbreaks in the population. The presence of hepatitis A virus (HAV) varies in different geographical areas, but it is frequently detected in urban sewage in endemic areas throughout the year. Hepatitis E virus (HEV), like HAV, is more abundant in countries where sanitation is poor. Autochthonous strains of HEV have been reported in urban sewage in several highly industrialized countries, as well as related cases of sporadic acute hepatitis caused by these non-imported strains (Pina et al., 2000; Clemente-Casares et al., 2003). Strains of HEV may also infect pigs, wild boar and deer, and they are frequently detected in both slaughterhouse sewage, where pigs are treated, and urban sewage in areas in Europe that were considered non-endemic (Rodriguez-Manzano et al., 2010).

Many of the viruses that are considered to be water contaminants produce primarily sub-clinical infections, causing symptoms only in a small proportion of the infected population. Enteroviruses are a good example, although they may cause a wide diversity of clinical syndromes, including diseases affecting the central nervous system. The syndromes produced by viral infections range from respiratory diseases, frequently associated with HAdV, to life-threatening conditions, such as acute hepatitis caused by HEV and HAV in adults. Other infections include severe gastroenteritis in small children and the elderly, commonly related to rotavirus, or adenovirus and norovirus respectively (Hart et al., 2009). Disease progression depends on the route of infection, the infective dose of the viral agent, the age, health, immunological, and nutritional status of the infected individual (pregnancy, presence of other infections or diseases), and the availability of health care.

Viruses that are transmitted via contaminated food or water are typically stable because they lack the lipid envelopes that render other viruses more susceptible to environmental agents and because survive in the digestive track. Some viruses, such as human polyomaviruses JC (JCPyV) and BK (BKPyV) and some HAdV, infect humans during childhood, thereby establishing persistent infections. In recreational water-borne diseases, noroviruses are believed to be the single largest cause of documented outbreaks, followed by adenovirus (Sinclair et al., 2009).

2.2. Bacteria

Salmonella and *Campylobacter* are the most frequent agents of bacterial gastroenteritis (Westrell et al., 2009). *Salmonella* is isolated from water in lower numbers than indicator bacteria such as faecal coliforms, faecal streptococci and enterococci, which are several orders of magnitude higher (Sidhu and Toze, 2009). However, low numbers (15–100 colony-forming units [CFU]) of *Salmonella* in water may pose a public health risk (Jyoti et al., 2009). Thermophilic *Campylobacter* species are widespread in the environment and are commonly found in surface water and sewage sludge (Sahlström et al., 2004). Other frequent water-borne pathogens are *Shigella*, *Yersinia* or *Vibrio cholerae*, with outbreaks linked to contaminated water and seafood (Sharma et al., 2003). *Legionella pneumophila* has a complex aquatic life cycle that strongly affects its state of activity. *L. pneumophila* is a ubiquitous bacterium in natural aquatic environments that can also persist in human-controlled systems containing water, such as air conditioning and plumbing infrastructures. Intracellular growth in protozoa can permit this pathogen to survive chlorination, and the generation of aerosols in these systems contributes to the transmission to humans where infection of alveolar macrophages results in respiratory illness (McDade et al., 1977; Steinert et al., 2002).

In addition to these well-known water-borne pathogens, there are groups of bacteria, some of them considered as "emergent pathogens" that are now also regarded as being transmitted by the water route. *E. coli* O157:H7 is a faecal pathogen frequently isolated from waters (Bavaro, 2009). It has been found in 2% of raw sludges, but the numbers of *E. coli* O157:H7 in sewage and its survival during wastewater treatment are unknown (Sidhu and Toze, 2009).

Listeria monocytogenes is a ubiquitous bacterium, isolated from a wide range of environmental sources, including soil, water, effluents, a variety of foods, and the faeces of humans and animals (Barbuddhe and Chakraborty, 2009). Recent outbreaks demonstrated that *L. monocytogenes* can also cause gastroenteritis in healthy individuals, and more severe invasive disease in immunocompromised patients (Barbuddhe and Chakraborty, 2009; Wilkes et al., 2009). *Vibrio vulnificus* is an opportunistic human pathogen that may cause gastroenteritis, severe necrotizing soft-tissue infections and primary septicaemia, with a high lethality rate. Illness is associated with ingestion of seafood or exposure to contaminated water. *V. vulnificus* has been recovered from fish, shellfish, water and sediments (Harwood et al., 2004). Recently, it has been isolated from wastewater samples (Igbinosa et al., 2009). *Helicobacter pylori* is an etiological agent of gastritis, and peptic and duodenal ulcers. In addition, infection is a recognized risk factor in the development of gastric mucosa-associated lymphoid tissue lymphoma and adenocarcinoma. *H. pylori* is present in surface water and wastewater (Queralt et al., 2005). Biofilms in drinking-water systems have been reported as possible reservoirs of *H. Pylori* (Park et al., 2001). Attempts to culture *H. pylori* cells from environmental water samples have been largely unsuccessful, and its ability to survive in an infectious state in the environment remains controversial. Due to the fastidious nature of the bacterium and the lack of standard culture methods for environmental samples, very few quantitative studies have been reported (Percival and Thomas, 2009). *Arcobacter* spp., which are considered to be emerging pathogens that cause diseases in domestic animals and diarrhoea in humans, are frequently isolated from animal food products, in particular from poultry, as well as various types of water such as groundwater, surface water, raw sewage and seawater (Ho et al., 2006). Various studies have concluded that water may also play an important role in the transmission of *Arcobacter* spp. and strongly suggest a faecal–oral route of transmission to humans and animals (González et al., 2007).

2.3. Protozoa

A number of different types of pathogens, such as *Cryptosporidium*, *Entamoeba*, *Cyclospora*, *Toxoplasma*, *Microsporidia* and *Giardia*, among others, can be present in contaminated water

(Smith et al., 2004). Among protozoan genera *Cryptosporidium* and *Giardia* are known to be highly resistant to environmental stress (Cacciò, 2003). *Cryptosporidium* has been detected in many drinking water sources and is considered an important water-borne contaminant (Xiao and Ryan, 2008). Infection occurs following ingestion of oocyst-contaminated food, drinking water, or recreational water. There are 20 valid *Cryptosporidium* species and over 40 genotypes of this parasite. Of these, *Cryptosporidium parvum* and *Cryptosporidium hominis* cause over 95% of the reported causes of human cryptosporidiosis (Xiao and Ryan, 2008).

Giardia is a frequent water contaminant. Of the morphologically defined *Giardia* species, only *Giardia duodenalis* (syn. *Giardia intestinalis* or *Giardia lamblia*), has been recovered from humans and a wide variety of other mammals (van der Giessen et al., 2006). The major sources of surface water contamination with *G. duodenalis* are discharges of treated or untreated sewage, run-off or discharges of manure from agricultural lands and, in more pristine waters, wildlife. Concentrations of cysts as high as 88,000 per litre in raw sewage and 240 per litre in surface water have been reported (Wallis et al., 1996). Cysts are robust and can survive for weeks to months in freshwater. *Cryptosporidium* spp. and *G. duodenalis* are common food- and water-borne protozoa that affect humans and a wide range of domestic and wild animals (Fayer, 2004). These parasites are among the major causal agents of diarrhoeal disease in humans and animals worldwide, and they can even potentially shorten the life span of immunocompromised hosts (Smith et al., 2007; Reynolds et al., 2008). *Giardia* spp. cysts are more resistant to chlorine than enteric bacteria but they are not as resistant as *Cryptosporidium*.

The number of parasites required to induce infection has been estimated to be as low as 10 *Cryptosporidium* spp. oocysts (Fayer et al., 2000) or 10 *G. duodenalis* cysts (Adam, 2001).

3. Available molecular techniques and applications

Culturing pathogens is a laborious procedure, involving enrichment and selective media in an attempt to isolate pathogens from background bacteria. It is often difficult to achieve appropriate enrichment, which makes the work even more tedious. Moreover, concentrations may be too low for cultural detection but still be high enough to cause infection. Therefore, a molecular detection method is needed, since such methods are highly specific and sensitive. The methods used are typically based on the detection and quantification of specific segments of the pathogen's genome (DNA or RNA). In order to reach the detection level, the specific segments are subjected to *in vitro* amplification. These methods allow researchers to rapidly and specifically detect microorganisms of public health concern. Additionally, recent improvements have allowed simultaneous detection of several microorganisms in a single assay (Maynard et al., 2005; Straub et al., 2005; Marcelino et al., 2006).

The range of protocols available for the application of molecular techniques has increased over the last few years, and the costs involved, although still significant, have fallen. These developments have allowed the potential standardization and automation of some of these techniques. In some cases they facilitate the identification, genotyping, enumeration, viability assessment, and source-tracking of human and animal contamination if host-specific highly prevalent pathogens are analyzed. Direct monitoring of pathogens has enjoyed wider application since the development of molecular technologies. However, the molecular techniques available today are being continuously refined in order to be standardized and make them applicable to a diversity of matrices, to increase their sensitivity, and to reduce the time and steps required in the analytical process.

The standardization and validation of protocols is considered a very important requirement for the implementation of molecular techniques either in the clinical or in the environmental field and has a major impact on the evaluation of the data produced in the diverse studies (Raymaekers et al., 2009; Bustin, 2010; Doring et al., 2008; Harwood et al., 2009; Sen et al., 2007). External evaluation programs for the detection and quantification of many pathogens using molecular methods are already being routinely established in many laboratories in the clinical field. Although several validation studies have been developed for water-borne pathogens using molecular methods (Conraths and Schares, 2006; Cheng et al., 2009) there is still the need for validation assays for most of the pathogens and indicators in water samples. These validation assays should involve diverse laboratories and selected methodologies applicable in standard protocols evaluating inter and intra-laboratory variability and providing robust information on the efficiency of the methods for a diversity of pathogens and matrices. The question of suitable controls has also been discussed in reference to PCR assays. In addition to the specific positive and negative controls in PCR reactions, other controls are required. Known quantities of DNA are used as internal or external controls in the reactions, and neat and diluted samples are tested to evaluate the presence of potential inhibitors that may affect the accuracy of the quantification. Also the use of an affordable process control will be required in order to demonstrate that the concentration and extraction protocols worked correctly for every assay.

Most methods utilize the following steps: (i) Concentration of the organism of interest from the environmental water sample into a suitable volume (if necessary); (ii) Extraction of the RNA or DNA from the target organism; (iii) Amplification of the genomic segment(s) chosen; (iv) Detection (or quantification) of the amplified genomic segment(s).

Most applied molecular techniques are based on protocols of nucleic acid amplification, of which the polymerase chain reaction (PCR) is the most commonly used. Quantitative PCR (qPCR) is rapidly becoming established in the environmental sector. qPCR is, in many cases, more sensitive than either the bacterial culture method or the viral plaque assay (He and Jiang, 2005). Quantitative reverse transcriptase PCR (qRT-PCR) uses RNA as a template molecule. qPCR commonly uses fluorescent dyes such as SYBR green for the detection of the amplified segment. However, molecular beacons or other fluorescent probes such as TaqMan assays (Applied Biosystems, Foster City, CA, USA), Scorpion primers (PREMIER Biosoft International, Palo Alto, CA, USA) or that used in the LightCycler (Roche, Indianapolis, Indiana, USA) lead to higher

specificity based on the use of complementary primers and probes for the quantification of the selected genome segment. The use of qPCR is extending, and is under consideration for monitoring the environment, water and food.

Besides PCR, other methods are available to amplify nucleic acids, for example Nucleic Acid Sequence-Based Amplification (NASBA), an isothermal method designed to amplify RNA from either RNA or DNA templates, although it is most commonly used to amplify RNA (Cook, 2003; Goodwin and Litaker, 2008).

3.1. *Molecular techniques for the analysis of viruses*

Practically all viral pathogens found in environmental waters – rivers, lakes, seawater and groundwater – originate from contamination with wastewater or directly with human or animal excreta. Most of the more than one hundred species of viruses of faecal origin cannot be detected with the conventional cell-culture methods, or their detection efficiency is very poor. For this reason, molecular methods for viruses in water have rapidly found their way in the analysis of the environment, and data are accumulating on the use of qPCR techniques to assess the presence and concentration of viruses in water. Even though PCR is a very sensitive detection technique, the amount of viruses found in many environmental waters is low and the viral particles must be subjected to a concentration step before the PCR can be attempted. A wide range of methodologies for the concentration and detection of viruses from water using molecular techniques have been tested. The techniques most commonly used are based on adsorption–elution-based virus concentration protocols with various filters or glass wool columns and also on ultrafiltration (Donaldson et al., 2002; Haramoto et al., 2005; Rajal et al., 2007; Lambertini et al., 2008; Albinana-Gimenez et al., 2009a). The concentration of viruses by adsorption-elution-based protocols is less efficient in seawater than in freshwater, perhaps as a result of the high ionic strength in seawater. Alternative techniques are based on direct flocculation methods (Calgua et al., 2008) or ultrafiltration procedures (Jiang et al., 2001). Examples of the concentrations of viruses that have been found in sewage, freshwater and seawater by diverse concentration procedures and molecular methods are presented in Table 1.

3.2. *Molecular techniques for the analysis of bacteria*

Classical microbiological quantification methodology relies on the cultivation of specific bacteria in appropriate culture media and on their further biochemical or immunological characterisation. For many well known and emerging pathogens, appropriate culture methods for environmental samples and biochemical schemes for valid identification at species level are lacking (Dong et al., 2008). On the other hand, target bacteria might be embedded in biofilms and not be accessible to the standard techniques. After prolonged exposure to water, bacterial pathogens might enter a viable but non-culturable stage, in which they cannot be detected by culture, although they retain their infective potential (Cenciarini-Borde et al., 2009). For these reasons, several studies have produced data on the quantification of bacteria in water by using qPCR techniques. However, molecular protocols, unlike traditional culture-based methods, do not distinguish between viable and non-viable organisms and although some approaches have been developed for discriminating damaged pathogens or naked nucleic acid from intact microorganisms, there is still the need for more information before replacing the current conventional methods by molecular ones.

Molecular techniques for the specific detection and quantification of bacterial pathogens also offer several advantages over conventional methods: high sensitivity and specificity, speed, ease of standardization and automation. As with the viruses, direct PCR amplification of some bacterial pathogens from water samples is difficult due to the presence of only low numbers of these bacteria in environmental sources. Therefore, an enrichment step is usually required prior to performing a PCR (Noble and Weisberg, 2005).

Quantitative PCR analysis of aquatic DNA provides the number of genomes per volume of water of a specific pathogen. This value is not identical with the number of cells that can be determined by microscopic techniques, such as fluorescent in situ hybridization (FISH) or immunofluorescence, or the CFUs obtained by plate counting. Bacterial pathogens can produce aggregates, many bacteria contain more than one genome per cell, depending on their physiological state, and also several of the gene markers used for detection, such as the rRNA genes, may be present in multiple copies in the bacterial genome. These features entail the application of different conversion factors from genome copies to cells for different pathogens, and such factors need to be determined for each targeted bacterial species individually (Brettar and Höfle, 2008). Examples of the concentrations of bacterial pathogens that have been found in water by qPCR are presented in Table 2.

The FISH technique, based on hybridization with rRNA oligonucleotide probes, has been used for the detection and identification of different microorganisms in mixed populations. This technique is considered a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology (Bottari et al., 2006). It helps to reveal mechanisms of survival and infection at the cellular level (Brettar and Höfle, 2008) and in the study of biofilms (Juhna et al., 2007). FISH has been applied with this aim for the detection of emerging pathogens from water, sewage and sludge (Gilbride et al., 2006). This method also allows for the detection of viable but non-culturable forms. However the methodology is still limited by a lack of sensitivity and enrichment steps are often required, either cultural pre-enrichment or a magnetic bead type enrichment, or both.

An example of multiplex PCR (mPCR) assays developed for the detection of bacterial pathogens are a multiplex SYBR green I-based PCR assay developed for simultaneous detection of *Salmonella* serovars and *L. monocytogenes* (Jothikumar et al., 2003). Another quadruplex qPCR assay for detection and differentiation of O1, O139, and non-O1, non-O139 strains of *V. cholerae* and for prediction of their toxigenic potential was developed by Huang et al. (2009). *L. monocytogenes* has also been investigated in biofilms using qPCR techniques (Guilbaud et al., 2005).

L. pneumophila is not transmitted through the oral route, however, it is also considered a water-borne pathogen and is the most commonly reported etiologic agent of legionellosis. It has an impact on public health in developed countries, as

Table 1 – Examples of the concentration of viruses found in sewage, freshwater and seawater by qPCR. Results are expressed in genome copy logs (GC logs).

Virus	Type of sample	Collection site	Concentration	% Positive samples	Quantification method	Reference
Adenovirus	Sewage (raw)	Spain	4–7 GC logs/100 ml	100%	qPCR	Bofill-Mas et al. (2006)
	Sewage (secondary effluent)		3 GC logs/100 ml	100%		
	Biosolids		4–7 GC logs/100 g	100%		
	River water		1–4 GC logs/l	90%		Albinana-Gimenez et al. (2009b)
	Seawater		1–3 GC logs/l			Calgua et al. (2008)
	Sewage (raw)	USA	4–5 GC logs/100 ml		qPCR	Fong et al. (2010)
	Sewage (tertiary effluent)		3–4 GC logs/100 ml			
Adenovirus 40, 41	River water	Japan	3–5 GC logs/l	61%	qPCR	Haramoto et al. (in press)
JC Polyomavirus	Sewage (raw)	Spain	5 GC logs/100 ml	100%	qPCR	Bofill-Mas et al. (2006)
	Biosolids		3–5 GC logs/100 g	100%		
	River water		0–3 GC logs/l	90%		Albinana-Gimenez et al. (2009b)
	Sewage (raw)	Brazil	4–7 GC logs/100 ml	96%	qPCR	Fumian et al. (in press)
	Sewage (secondary effluent)		4–5 GC logs/100 ml	39%		
	River water	Japan	2–3 GC logs/l	11%	qPCR	Haramoto et al. (in press)
Astrovirus	Sewage (raw)	France	5–7 GC logs/100 ml	100%	qRT-PCR	Le Cann et al. (2004)
Enterovirus	Sewage (raw)	France	7 GC logs/100 ml		qPCR	Schvoerer et al. (2001)
Hepatitis A virus	Sewage (raw)	Spain	4 GC logs/100 ml		qPCR	Rodriguez-Manzano et al. (2010)
Hepatitis E virus	Sewage (raw)	Spain	3 GC logs/100 ml		qPCR	Rodriguez-Manzano et al. (2010)
Norovirus	Sewage (raw)	United Kingdom	6 GC logs/100 ml		qPCR	Laverick et al. (2004)
GII	Sewage (effluent)	Brazil	2–3 GC logs/l		qPCR	Victoria et al. (in press)
GI			2 GC logs/l			

demonstrated by the many major outbreaks reported over the past years. Molecular techniques based on qPCR have been described for the detection of pathogenic strains of *L. pneumophila* (Morio et al., 2008). Due to the ubiquitous nature of *L. pneumophila* bacterium in the environment, molecular typing methods are needed both to determine the relatedness of outbreak strains and to identify the source of the outbreak.

3.3. *Molecular techniques for the analysis of protozoa*

The identification of *Cryptosporidium* and *Giardia* (oo)cysts in environmental samples is largely achieved by the use of immunofluorescent assays (IFAs) after concentration processes using U.S. EPA method 1623 and its equivalents in other countries (USEPA, 2001). These microscopic-based methods produce total counts of live and dead *Cryptosporidium* oocysts in water samples, without distinguishing species or genotypes that can infect humans from those that cannot (Brescia et al., 2009). There are currently four methods by which oocyst viability can be assessed including (Millar et al., 2002): (i) animal infectivity, (ii) *in vitro* excystation, (iii) the exclusion/inclusion of vital fluorogenic dyes, and (iv) Reverse transcriptase PCR (RT-PCR).

Given the limitations in the specific detection of *Cryptosporidium* and *Giardia* using microscopy, immunological and/or flow cytometric methods, a range of nucleic acid-based methods have been developed and evaluated for the identification of species, the detection of genetic variation within and among species from faecal, environmental or water samples, and the diagnosis of cryptosporidiosis and giardiasis. Some methods rely on the *in situ* hybridization of probes to particular genetic loci within *Cryptosporidium* oocysts or *Giardia* cysts, whereas most rely on the specific amplification of one or more loci from small amounts of DNA by the PCR. The introduction of molecular techniques, in particular those based on the amplification of nucleic acids, has provided researchers with highly sensitive and specific assays for the detection and quantification of protozoa. Because *Giardia* and *Cryptosporidium* usually occur in low concentrations, detection methods should also include a procedure for concentrating the organisms from large-volume water samples. Filtration, flocculation, flow cytometry, immunomagnetic separation (IMS) and immunofluorescence with monoclonal antibodies together constitute the currently accepted methodology for detecting protozoa in drinking water (Slifko et al., 2000). These approaches are also used for testing raw and treated waters, although PCR-based procedures are increasingly being used in quality control (Thompson et al., 2004). In addition, molecular techniques can provide genotypic characterisation of the parasites isolated from water, thus helping to identify the

Table 2 – Examples of the concentration of bacterial pathogens found in water by qPCR. Results are expressed as mean values of genome copy logs (GC logs) or cell logs. It must be considered that most data is approximate because some of the studies had only one or two positive results or were tested after rainfall events or by using standard curves of different bacterial strains.

Bacteria	Quantitative assay	Type of samples	Concentration	Reference
E. coli	Taq-Man	Sewage (raw)	7 GC logs/100 ml	Shannon et al. (2007)
		Sewage (effluent)	3 GC logs/100 ml	
Enterococos	Taq-Man	Lake water	3 GC logs/100 ml	Lavender and Kinzelman (2009)
	Taq-Man	Sewage (raw)	4 GC logs/100 ml	Shannon et al. (2007)
		Sewage (effluent)	3 GC logs/100 ml	
Salmonella	SYBR green	Surface water	2 GC logs/100 ml	Ahmed et al. (2009)
Campylobacter jejuni	SYBR green	Surface water	1 GC logs/100 ml	Ahmed et al. (2009)
Arcobacter sp.	SYBR green	Sewage (raw)	2–6 cell logs/100 ml	González et al. (unpublished)
Enterohemorragic *E. coli*	SYBR green	Surface water	14/32 positives samples, levels under 10 GC/100 ml (assay quantification limit)	Ahmed et al. (2009)
V. vulnificus	Taq-Man	Seawater (estuarine)	Between 2 and 91 cells/100 ml	Wetz et al. (2008)
L. monocytogenes	Taq-Man	Sewage (raw)	3 GC logs/100 ml	Shannon et al. (2007)
		Sewage (effluent)	Absence	
H. pylori	SYBR green	Sewage	2–3 GC logs/100 ml	Nayak and Rose (2007)
Clostridium perfringens	Taq-Man	Sewage (raw)	5 GC logs/100 ml	Shannon et al. (2007)
		Sewafe (effluent)	3 GC logs/100 ml	
V. cholera, *Shigella dysenteriae*, *Salmonella thyphimurium* and *E. coli*	SYBR green	River water (low level of pollution)	1.8 GC logs/100 ml	Liu et al. (2009)
		River water (high level of pollution)	3.7 GC logs/100 ml	
		2 Urban lakes	2.7–3.3 GC logs/100 ml	

source of contamination (Thompson et al., 2004). Molecular tools have been developed to detect and differentiate *Cryptosporidium* at the species/genotype and subtype levels (Xiao and Ryan, 2004; Cacciò, 2005), enabling researchers to perform more accurate risk assessment of environmental and drinking water contamination (Xiao and Ryan, 2008). Recently, a two-color FISH assay, based on species-specific probes for *C. parvum* (Cpar 677 probe) and *C. hominis* (Chom253 probe), has been shown to distinguish between the two major species involved in human infections (Alagappan et al., 2009). The FISH assay is subject to the same limitations of sample recovery efficiency and purification losses that affect other methods (Jakubowski et al., 1996) and also the physiological condition of the (oo)cysts affects the fluorescence intensity of FISH-positive (oo)cysts.

Quantitative RT-PCR is used to detect messenger RNA (mRNA), which is present only in viable organisms since its stability is short, often only a few seconds to minutes (Baeumner et al., 2001). Therefore, mRNA is an optimal target molecule if viable organisms need to be distinguished from non-viable organisms. However, not all mRNAs are produced throughout the life of an organism (Baeumner et al., 2001). qPCR protocols have been developed for the detection and identification of *Cryptosporidium* and *Giardia* species/genotypes from water samples. Examples of the molecular targets and sensitivities of the assays that have been used in water samples are presented in Table 3. Finally, other molecular assays, such as NASBA assays, have also been described for the detection of some bacterial pathogens and parasites in the environment and food (Baeumner et al., 2001; Cacciò, 2003; Cook, 2003; Thompson et al., 2003).

The quantification of protozoa abundance in water by molecular methods has been described by several authors. Guy et al. (2003) detected cyst concentrations ranging from 2653 to 13,408/l in sewage samples from wastewater treatment plants in Laval (Quebec) with TaqMan probe qPCR. Anceno et al. (2007) detected cyst concentrations ranging from 243 to 4103/l with SYBR green qPCR in an irrigation canal receiving discharges from a wastewater treatment plant in the periurban area of Thailand. Bertrand et al. (2004) detected cyst concentrations ranging from 250 to 2300/l in sewage influent samples from a wastewater treatment plant in Nancy (France) with qPCR.

4. Human and animal viruses as faecal indicators and MST tools

The high stability of viruses in the environment, host-specificity and the high prevalence of some viral infections throughout the year in the world population strongly support the use of qPCR techniques for the identification and quantification of specific viruses that can be used as indicators of faecal contamination and as MST. HAdV and the JCPyV have been suggested as indicators of human faecal contamination, given their high prevalence in all the geographical areas studied as indicators of human faecal contamination (Puig et al., 1994; Pina et al., 1998; Bofill-Mas et al., 2000). The identification of faecal microbial contamination and their sources in the environment, water and food, plays a very important role in enabling effective management and remediation strategies. MST includes a group of methodologies that aim to identify, and in some cases quantify, the dominant sources of faecal contamination in the environment and, more specifically, in water resources (Field, 2004; Fong and Lipp, 2005). Molecular methods based on molecular detection of host-specific strains of bacteria from the

Table 3 – qPCR assays for the detection and quantification of *Cryptosporidium* and *Giardia* in environmental samples. The genomic targets, the pathogen(s) detected, the sensitivity, and the nature of the investigated samples are indicated (adapted from Cacciò, 2003).

Type of assay	Genomic target	Pathogen(s) detected	Sensitivity	Nature of the sample	References
TaqMan	Oocyst wall protein gene (COWP)	*C. hominis*, *C. parvum*, *Cryptosporidium meleagridis*, *Cryptosporidium wrairi*	1 Oocyst	Environmental samples (water and sewage)	Guy et al. (2003)
TaqMan	Anonymous fragment (AF118110)	*C. hominis*, *C. parvum*, *C. meleagridis*	8 Oocysts	River water	Fontaine and Guillot (2003)
TaqMan	β-giardin	*G. duodenalis* (Assemblages A and B)	1 Cyst	Environmental samples (water and sewage)	Guy et al. (2003)
TaqMan	Elongation factor 1A	*G. duodenalis*, *Giardia ardae*	1 Cyst	Wastewater	Bertrand et al. (2004)
Quenching probe	18S rRNA	*Cryptosporidium* spp.	1 Oocyst	River water	Masago et al. (2006)
TaqMan	Triose-phosphate isomerase	*G. duodenalis* (Assemblages A and B)	3 Cysts Assemblage A 4.5 cysts Assemblage B	Wastewater	Bertrand and Schwartzbrod (2007)

Bacteroidales order and the genus *Bifidobacterium* (Bernhard and Field, 2000a,b; Harwood et al., 2009; Dorai-Raj et al., 2009) have been widely used. The quantification by PCR of DNA viruses has some advantages in relation to the quantification of RNA viruses, such as lower cost and lower sensitivity to inhibitors potentially present in the sample. In a study by Harwood et al. (2009), three laboratories evaluated library-independent MST methods for human sewage detection via conventional PCR: human-associated Bacteroidales, human polyomaviruses, and *Methanobrevibacter smithii*, and the results showed that human polyomaviruses were the most specific human marker for MST.

The evaluation of the correlation between indicators and specific pathogens would require further studies. It has been shown that HAdV were the viruses most frequently detected throughout the year, and most of the samples that were positive for enteroviruses or HAVs were also positive for HAdV (Pina et al., 1998). The presence of human adenovirus or JCPyVs in water is an indication of faecal/urine contamination and potential risk for the presence of faecal/urine pathogens. However, the excretion patterns of some specific pathogens such as noroviruses or rotaviruses are different, and their dissemination as environmental contaminants in water change depending of the period of the year (higher in cold months in temperate areas) and the potential presence of outbreaks in the population. It is then clear that human adenoviruses and polyomaviruses may be considered a useful marker of human contamination but it is also clear that, in some cases and specific locations, the numbers of viruses related to specific outbreaks, such as rotaviruses, may exceed the numbers of human adenovirus in this specific environment (Miagostovich et al., 2008).

Specific animal viruses have also been proposed as MST tools such as porcine adenoviruses (PAdV) and bovine polyomaviruses (BPyV) (Bofill-Mas et al., 2000, 2006; Formiga-Cruz et al., 2003; Hundesa et al., 2006; Hundesa et al., 2009a,b), bovine enterovirus and teschoviruses (Jimenez-Clavero et al., 2003, 2005). PAdVs and BPyV are disseminated widely in the swine and bovine population respectively but they do not produce clinically severe diseases. A summary of the concentration values reported for BPyV and PAdV in environmental samples is shown in Table 4.

5. Molecular techniques in the microbiological control of water quality: The pros and cons

PCR techniques such as qPCR and qRT-PCR using specific probes, with a high level of sensitivity and specificity, represent rapid, cost-effective tools that generate significant information on the presence, quantity and distribution of classical and new emergent pathogens in water and food. For this reason molecular technologies for water quality analysis are widely used for the identification of pathogens in water. The final goal of molecular-based technologies is not simply to speed up slow culture methods, but to achieve an increase in the accuracy and sensitivity of the detection methods. To this end, great attention is being paid to the robustness of the

Table 4 – Results obtained in quantitative PCR (qPCR) assays for Bovine polyomaviruses (BPyV) and Porcine adenoviruses (PAdV) in different types of environmental samples. Results expressed as genome copies (GC) per l. qPCR assays were performed according to Hundesa et al. (2009a,b).

Type of samples	No. of samples	Levels of BPyV and PAdV in the samples analyzed					
		BPyV			PAdV		
		% Of positives samples	Mean values	Standard deviation	% Of positive samples	Mean values	Standard deviation
Slaughterhouse wastewater (GC/l)	10	90.9	2.84×10^3	4.87×10^3	100	1.56×10^6	1.18×10^6
Ter river water (GC/l)	6	50	3.06×10^2	2.03×10^2	100	8.38	6.11

assays (Signoretto and Canepari, 2008). The main pros and cons of molecular techniques are as follows.

5.1. *The pros of molecular techniques applied to the detection of pathogens in water*

- For many pathogens and proposed new indicators, molecular techniques are the only method of detection and quantification because there is no efficient culture system.
- Molecular techniques are used as identification tools for specific strains, genotyping and confirmation of the results. PCR and sequence analysis provide further information on the phylogenetic characteristics of the strains identified.
- The qPCR methodology facilitates the evaluation of the efficiency of removal of pathogens or selected indicators in water treatment plants, including viruses. It is also a very useful tool for the identification of the sources of faecal contamination in water.
- PCR is a powerful tool in risk assessment. The qPCR and qRT-PCR techniques provide quantitative estimations of the concentration of pathogens in water. qPCR and nucleic acid extraction protocols are easy to standardize and automate, and they can be used in the detection of pathogens that may be present below the limit of detection of other assays. The high numbers of samples that can be processed in this manner allow, in combination with epidemiological surveys, to carry out risk assessment studies that would otherwise be difficult to accomplish.

5.2. *The cons of molecular techniques applied to the detection of pathogens in water*

- The detection of genomes by PCR-based techniques does not provide information about the infectivity of the pathogen or the indicator detected or the level of risk for the population. Disinfection of water by UV and chlorine treatments reduces the numbers of viral particles quantified by qPCR and qRT-PCR if severe treatments are applied. However, commonly applied disinfection treatments produce a significant reduction in the number of infectious viral particles without showing equivalent variations in the level of viral genomes quantified by qPCR and qRT-PCR.
- The protocols used need to be improved and standardized. In the case of protocols for the concentration of viruses from water samples, differences of more than 2 logarithms are observed when using distinct concentration methods and qPCR (Albinana-Gimenez et al., 2009a). Fig. 1 shows the intra-laboratory variability for testing different methods of concentration of viruses in artificial seawater and freshwater. The methods tested are based on membrane concentration methods with different eluents, electropositive filters, glass wool columns and direct flocculation (Sobsey and Glass, 1980; Katzenelson et al., 1996; Pallin et al., 1997; Vilaginès et al., 1997; Calgua et al., 2008). Moreover, the specific PCR conditions, primers and probes used may produce significant differences in the results (Bofill-Mas et al., 2006).
- The presence of inhibitors for the molecular assays in the samples still represents a limitation in the analysis of environmental samples. Nucleic acid extraction efficiencies vary considerably between different methods and the final nucleic acid yield depends on the methods used and the type of environmental sample. This makes direct comparison of absolute gene numbers between studies extremely problematic. Furthermore, the concentration at which inhibitors no longer affect the qPCR for any sample is not known *a priori* and must be determined empirically to ensure that the environmental template and the target gene for the standard curve have equivalent amplification efficiencies (Smith and Osborn, 2009). The efficiency of the reverse transcription may also be variable and in general qRT-PCR is considered to be more sensitive to inhibitors than qPCR.

6. Viability assays and molecular detection methods

Water treatment procedures aim to kill pathogenic bacteria and protozoa, and it is clear that their physiological state in water is a major issue for water safety (Brettar and Höfle, 2008). To assess whether viable cells, and not only DNA, are detected in the samples, molecular methods must be adapted. Various approaches have been evaluated, such as DNA-intercalating dyes like ethidium monoazide (EMA) and propidium monoazide (PMA) to selectively remove cells with compromised membranes from the analysis. These dyes enter a cell and bind covalently to DNA when photo-activated. PCR amplification of such modified DNA is strongly inhibited. PMA has been used to discern whether a cell is alive or dead, in combination with qPCR (Nocker and Camper, 2009). A limitation of this technique, however, is that the principle is based on membrane integrity as the viability criterion. Another

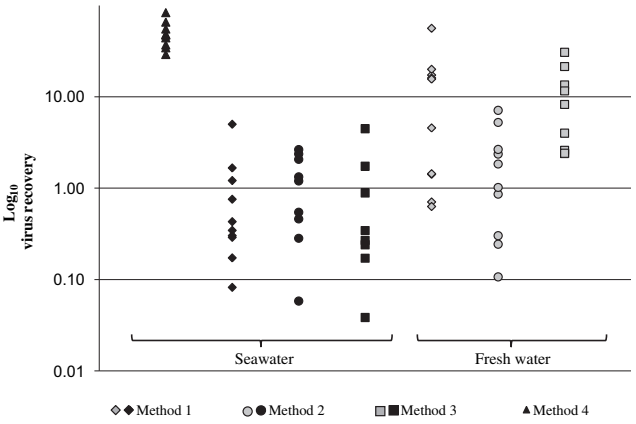

Fig. 1 – Intra-laboratory variability of viral concentration methods in artificial seawater and freshwater. Virus recovery values obtained after spiking sets of ten 10-l samples with HAdV 2, concentrating by: Method 1: electronegative filters of nitrocellulose and glycine 0.05 M pH 9.5 – skimmed milk buffer. Method 2: electronegative filters of nitrocellulose and glycine 0.25 M pH 9.5 – beef extract buffer. Method 3: a column of glass wool and glycine 0.25 M pH 9.5-beef extract buffer. Method 4: Direct organic flocculation with skimmed milk and quantifying the recovery by qPCR according to Bofill-Mas et al. (2006).

Table 5 – Quantification of Human adenovirus (HAdV 2 and 41) and JC polyomavirus (JCPyV) viral suspensions evaluated by immunofluorescence assay (IFA) and quantitative PCR (qPCR) with previous DNAse treatment. Results expressed as genome copies (GC) or focus forming units (FFU) per ml of suspension. qPCR assays were performed according to Bofill-Mas et al. (2006).

Virus	Viral suspension	DNAse treatment	qPCR	IFA
			GC/ml	FFU/ml
HAdV 2	1	+	2.70×10^5	1.60×10^4
		–	NT[a]	
	2	+	1.25×10^5	1.15×10^4
		–	$4.1E \times 10^5$	
HAdV 41	3	+	5.00×10^5	1.40×10^4
		–	NT	
	4	+	5.10×10^5	1.70×10^4
		–	7.17×10^6	
JCPyV	5	+	5.00×10^5	1.40×10^4
		–	NT	
	6	+	2.35×10^5	1.31×10^4
		–	9.64×10^6	

a NT: not tested.

possible solution is to target mRNA, as it tends to degrade relatively rapidly after cell death. However, there is increasing evidence that in many cases, mRNA persistence depends on the genes targeted and the conditions in which the cells were inactivated, and some RNA molecules can also persist in cells after loss of viability. Although weaker than that of DNA, this persistence of mRNA can, for example, lead to false positive results in the first hours after cell death when monitoring disinfection efficacy. Moreover, some mRNA molecules are not transcribed in the viable non-culturable (VBNC) state. Additionally, precautions must be taken to avoid the contamination with genomic DNA when RT-PCR is performed. Thus, it is currently impossible to develop a general protocol, and mRNA detection methods can be used only for specific study cases (Cenciarini-Borde et al., 2009).

A new CryptoPMA-PCR assay, which allows genotyping and viability determination, may improve the data on water-borne exposure to *Cryptosporidium* and enhance the validity of human risk assessment (Brescia et al., 2009). The use of qPCR with PMA treatment may also enable quantization of viable pathogenic *Cryptosporidium* oocysts in environmental samples (Brescia et al., 2009).

Diverse studies have been also developed in order to approach the molecular detection assays to the infectivity of the viral particles present in the analyzed samples. Nuanualsuwan and Cliver (2002) treated with RNAse and Proteinase K viral suspensions of hepatitis A, poliovirus vaccine 1 and feline calicivirus after inactivation by heat, ultraviolet light or hypochlorite as a procedure for detecting only structured particles. Data on the stability of HAdV and JCPyV using qPCR with and without a DNase treatment before nucleic acid extraction indicate that viral DNA is not stable in urban sewage for long periods (Bofill-Mas et al., 2006). The t_{99} (time required to observe a reduction of 99% in the initial viral concentration) has been calculated from regression curves as 132.3 days for HAdV and 127.3 days for JCPyV at 20 °C. When the viral concentrate was treated with DNase previously to the nucleic acid extraction, the t_{99} observed was 126.1 days for HAdV and 121.4 days for JCPyV. As expected, these viruses were more stable in phosphate buffered saline (PBS) than in sewage. Tables 5 and 6 report the quantification of viruses in viral suspensions, purified from cell-culture supernatants, and also of viruses in seawater, using cell-culture techniques, qPCR and qRT-PCR with and without DNAse or RNAse treatments.

7. Future developments

The application of new technologies such as high-throughput mass sequencing to analyze stool samples collected from patients with acute diarrhoea, and the use of nested-PCR (nPCR) and nucleotide sequence analyses in the study of faecal and environmental samples has greatly increased the number of viruses that can be identified in the environment. For example, a new picornavirus has recently been identified in gastroenteritis patients, and sewage, Klassevirus I (Holtz et al., 2008, 2009; Blinkova et al., 2009), and it is expected to produce interesting new information in the future.

Table 6 – Quantification of Human Adenovirus 2 (HAdV 2) and Murine Norovirus 1 (MNV1) in natural and artificial seawater spiked with both viruses. Virus concentration was evaluated at 0 and 60 min by plaque Assay, qPCR and qRT-PCR with previous enzymatic treatment (RNase for MNV1 and DNase for HAdV2 respectively). Results expressed as genome copies (GC) or plaque forming units (PFU) per ml. qPCR assays were performed according to Baert et al. (2008) and Bofill-Mas et al. (2006). Mean values of two replicate experiments are showed.

Virus	Seawater	DNAse	GC/ml (t_0/t_{60})	PFU/ml (t_0/t_{60})
HAdV 2	Natural	+	$2.3 \times 10^5/1.00 \times 10^5$	$2.50 \times 10^4/1.80 \times 10^4$
		–	$1.71 \times 10^7/1.65 \times 10^7$	
	Artificial	+	$4.02 \times 10^5/3.47 \times 10^4$	$1.65 \times 10^4/1.40 \times 10^4$
		–	$1.02 \times 10^7/1.90 \times 10^6$	
		RNAse	GC/ml (t_0/t_{60})	PFU/ml (t_0/t_{60})
MNV1	Natural	+	$1.40 \times 10^8/2.00 \times 10^7$	$1.95 \times 10^5/1.40 \times 10^5$
		–	$4.99 \times 10^8/7.65 \times 10^8$	
	Artificial	+	$7.00 \times 10^7/1{,}03 \times 10^8$	$3.95 \times 10^5/2.90 \times 10^5$
		–	$9.21 \times 10^8/7.54 \times 10^8$	

Several authors have suggested developing integrated systems for detecting multiple pathogens and indicators in source, drinking and recreational water. DNA microarray technologies could be the basis for such a test, although initial results have shown that direct hybridization of genomic DNA or RNA may not have the desired sensitivity. If microarray technologies could be coupled with PCR amplification of the target genes the signal sensitivity could be increased by 10^6-fold (Gilbride et al., 2006).

Other technical improvements are to be expected as a result of the advances in microfluidics and nanobiotechnology. Miniaturized systems could be based on microchips, and several approaches have been described (Ivniski et al., 2003; Gilbride et al., 2006).

More information, on the stability of genetic markers and distribution of pathogens and indicators in diverse geographical areas and the diverse matrices is needed for the identification of the most suitable molecular targets for detection and quantification of pathogens and the evaluation of the applicability of new indicators and MST tools. More research is required on the identification of indicators that better correlate to pathogen presence as even the newly emerging indicators in MST development often have poor success in predicting pathogen presence. However, the epidemiological pattern of many pathogens is different which makes it necessary to distinguish between the significance of analyzing widely spread, highly prevalent indicators of faecal/urine contamination in water as an indication of potential contamination by many of the pathogens and the detection of specific pathogens that may be sporadically highly abundant in water and often do not correlate to other indicators. Several assays based on molecular techniques for detection and quantification of pathogens and potential indicators have been developed that may be validated and standardized, and the technology could be ready for routine implementation and automation in the near future.

8. Conclusions

Molecular techniques, specifically nucleic acid amplification procedures, provide sensitive, rapid and quantitative analytical tools for studying specific pathogens, including new emergent strains and indicators. They can be used to evaluate the microbiological quality of water, the efficiency of pathogen removal in drinking and wastewater treatment plants, and in MST studies.

Water disinfection treatments may have an effect on the viability of pathogens and the application of molecular techniques produces numbers of genome copies that may overestimate the concentration of infectious microorganisms.

The molecular techniques available today and those under development would require further refinement in order to be standardized and applicable to a diversity of matrices.

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